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13. ABSTRACT *(Maximum 200 words)*

Under the auspices of this training Fellowship (May 9, 1994-November 9, 1997) I have undertaken studies on the molecular mechanisms that govern ligand-induced endocytosis of epidermal growth factor receptors (EGFR) and the role of regulated endocytosis of EGFR in controlling EGFR signaling. To this end, I developed a novel cell-free assay which reconstitutes the ligand-induced and EGFR tyrosine kinase-dependent recruitment of EGFR into clathrin coated pits. I also showed that activation of small Rho-family GTPases, which occurs in response to growth factors, can negatively regulate receptor-mediated endocytosis. In following up this discovery I have provided new and direct evidence for the involvement of the actin cytoskeleton in early events in receptor-mediated endocytosis. Finally, I have begun to explore the role of an EGFR tyrosine kinase substrate, Eps15, in receptor-mediated endocytosis. Eps15 is the prototype member of a growing family of EH (Eps15-homology) domain-containing proteins required for intracellular membrane trafficking. This successful training period has resulted in five manuscripts published in first-rate journals and one manuscript currently under review. It has paved the way for my continuing career objectives in cancer and medical research.

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FOREWORD

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December 4, 1997

Judy Pawlus
Chief, Research Data Management
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504 Scott Street
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Dear Dr. Pawlus:

The following is our final report for Gant Number DAMD17-94-J-4031. It describes work done by Dr. Christophe Lamaze during his training as a Postdoctoral Fellow in my laboratory. You will see that Dr. Lamaze was extremely productive and successfully completed his Postdoctoral Training. Work under the auspices of this Fellowship resulted in 5 published manuscripts and an additional manuscript currently under review. These manuscripts appeared in the highest quality journals including one in Nature and one in Science.

In the past, our annual reports have been criticized, not for the quality of the work, but for its divergence from the original Statement of Work. I would remind the referees that this grant was originally funded as a Postdoctoral Fellowship, for the stated purpose of "Training and Recruitment". I strongly believe that training was my highest obligation and that this main goal has been successfully fulfilled. Dr. Lamaze has spoken at both National and International Meetings of the Cell Biology Society and is currently being considered for a permanent INSERM position in France. He is a talented young investigator who has acquired, as a result of this training fellowship, the tools needed to establish his own successful career. Dr. Lamaze's initial degree was in Pharmacy and he continues his high interest in Medical Research.

I would like to take this opportunity to thank the Army for its support of Dr. Lamaze's training. I strongly believe that these relatively small investments in training will have, in the long run, the largest pay-off in terms of medical advances and human welfare as this new and enthusiastic generation of scientists pursue their research objectives.

Sincerely yours,

Sandra L. Schmid, Ph.D.
Principal Investigator

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INTRODUCTION

Epidermal Growth Factor (EGF) is the paradigm of peptide growth factors that bind to receptor tyrosine kinases (in this case, to the EGF-receptor) on the cell surface to trigger signalling events leading to cell proliferation and differentiation. A link between EGF physiopathology and breast cancer tumorigenesis has been clearly established. The binding of EGF to its receptor is followed by the rapid recruitment of activated receptor tyrosine kinases into coated pits, their internalization via coated vesicles and their delivery to the endosomal-lysosomal compartments for degradation. Ultimately, these triggered membrane trafficking events lead to a decrease in the number of EGF receptors expressed at the cell surface, a process termed receptor down-regulation. Over the past three years, we have developed and utilized a novel cell-free system to identify and purify new factors controlling the recruitment of activated EGF receptors into coated pits (Lamaze et al., 1993; Lamaze and Schmid, 1995) and to mechanistically characterize the molecular events involved. Last year, we provided the first evidence that the recruitment of activated EGF receptors into coated pits was indeed critical for the control of the mitogenic activity of EGF (Vieira et al., 1996).

In this Final Report, we summarize our research described earlier in previous Annual Reports (*section a*), and then briefly describe our studies published during this final funding period which report our initial characterization of the role of endocytosis in EGFR signalling (Vieira et al., 1996) and our discovery of a role for the actin cytoskeleton in receptor-mediated endocytosis (Lamaze et al., 1997) (*sections b-c*). Preliminary results directed towards some of these studies were reported in last year's Annual Report, as were new results related to analysis of Eps15, an EGFR tyrosine kinase substrate recently implicated in endocytosis. These new findings led to our submission of a revised Statement of Work (submitted 10/96). The bulk of this report, then, describes new results from our studies of the role of Eps15 in the recruitment of EGF and transferrin (Tfn)-receptors into coated pits.

Eps15 is tyrosine phosphorylated by the EGF-R (Fazioli et al., 1993; Schumacher et al., 1995), and associates with activated EGF-R (van Delft et al., 1997). It has been shown that Eps15 is constitutively bound to the AP2 constituents of the endocytic clathrin coat through its C-terminal proline-rich domain, designated DIII (Benmerah et al., 1995, 1996). Based on these findings it has been proposed that Eps15 may function coordinately with AP2 molecules as adaptors involved in EGF-R recruitment into coated pits. Indeed, our preliminary results had shown that the DIII of Eps15 was a strong inhibitor of the recruitment of EGF receptors into coated pits in perforated cells. Therefore, we initiated a collaboration with the Bensussan group to test the role of Eps15 in endocytosis of the EGF-R. The results from this collaboration respresent the bulk of this report (*section d*).

EXPERIMENTAL METHODS , RESULTS AND DISCUSSION

a) Ligand-induced EGFR endocytosis in a cell-free system

We have developed a cell-free assay system that measures the ligand-induced endocytosis of EGFR into coated pits (Lamaze et al., 1993). Under the auspices of this grant, we used this assay to provide the first direct biochemical evidence for a role for EGFR tyrosine kinase activity in ligand-induced endocytosis of activated EGFR (Lamaze and Schmid, 1995). In these studies, we also showed that a soluble and constitutively

active EGFR tyrosine kinase domain can support ligand-induced endocytosis of kinase-defective mutant EGFR *in trans*. This provided a functional assay for the necessary kinase substrate. The isolation and identity of this substrate is being pursued by other members of the Schmid lab currently supported by a grant from the National Cancer Institute (CA69099).

b) The role of endocytosis in regulating EGFR signalling .

As described in last year's report, we have analyzed EGF receptor (EGFR) signalling in mammalian cells conditionally defective for receptor-mediated endocytosis. We found that EGF-dependent cell proliferation was enhanced in endocytosis-defective cells, consistent with previous studies on cells expressing mutant endocytosis-defective EGFR, suggesting that endocytosis was essential for the down-regulation and attenuation of EGFR signalling (Wells et al., 1990). However, using this novel approach of studying the signalling from endogenous wild-type EGFR in cells we discovered that not all early EGF-dependent signalling events were uniformly upregulated. Instead, we found that activation of a subset of signal transducers required the normal endocytic trafficking of EGFR. These results are summarized in Table 1. Specifically, we found that tyrosine phosphorylation of SHC and PLCγ were increased in endocytosis defective cells consistent with the increased cell proliferative response. However, tyrosine phosphorylation of rasGAP, p85/PI3-kinase and of the MAP kinases, ERK1 and ERK2 as well as activation of MAP kinase were reduced in endocytosis-deficient cells. Thus, we have shown that endocytic trafficking of activated EGFR plays a critical role, not only in attenuating EGFR signalling, but also in establishing and controlling specific signalling pathways (Vieira et al., 1996, see appendix).

Table 1: Summary of altered EGFR signalling events in cells defective in endocytosis

Signalling events increased in endocytosis-deficient cells	Signalling events decreased in endocytosis-deficient cells
cell proliferation	EGF-R tyrosine phosphorylation
^{3}H-thymidine incorporation and cell proliferation (MTT assay)	ERK 1 and ERK 2 tryosine phosphorylation and MAP kinase activity
PLCγ tryosine phosphorylation	rasGAP tryosine phosphorylation
Intracellular Ca^{2+} levels	p85/PI3-kinase tryosine phosphorylation

c) A role for the actin cytoskeleton in receptor-mediated endocytosis

Last year we reported our findings (Lamaze et al., 1996) that activated Rho-family GTPases inhibit receptor-mediated endocytosis in mammalian cells. Rho-family GTPases are activated in response to growth factors, such as EGF, and so this negative regulation of endocytosis migth play provide a positive feed-back mechanism for maintaining activated EGF-R signalling complexes on the cell surface. Rho-family GTPases are known to regulate assembly and disassembly of the cortical actin cytoskeleton and therefore these results implied a role for actin in endocytosis. While it is clear that actin filament organization is essential for endocytosis in yeast ((Kubler and Riezman 1993; Munn, Stevenson et al. 1995)), a role for actin in mammalian cell endocytosis had not been established. For example, cytochalasin D, an actin-

depolymerizing drug, has no effect on receptor-mediated endocytosis either in nonpolarized mammalian cells or from the basolateral surface of polarized MDCK cells. Given this disparity, we re-examined the role of actin filaments in receptor-mediated endocytosis using highly specific reagents known to sequester actin monomers. Briefly, our published findings (Lamaze et al., 1997, see appendix) established that thymosin β4 and DNase I potently inhibited the sequestration of Tfnn and EGF receptors into coated pits as measured in a cell-free system using perforated A431 cells. At low concentrations, thymosin β4 but not DNaseI was stimulatory. Importantly, the effects of both reagents were specifically neutralized by addition of actin monomers. A role for the actin cytoskeleton was also detected in intact cells where latrunculin B, a drug that sequesters actin monomers, inhibited receptor-mediated endocytosis. These results provided new evidence that the actin cytoskeleton is required for the formation of endocytic coated vesicles in mammalian cells.

d) Role of Eps15 COOH-terminal domain in Tfn and EGF endocytosis

Although Eps15 is phosphorylated in response to EGF it is unclear what role this phosphorylation plays in regulating Eps15 function. For example, stimulation of cells with EGF treatment does not effect either the localization of Eps15 to coated pits (van Delft et al., 1996) or its constitutive association with AP-2 complexes (Benmerrah et al., 1995). Based on these observations, we initiated a collaboration with N. Bensussan to examine the role of Eps15 in both constitutive endocytosis of transferrin (Tfn) and ligand-induced endocytosis of EGF. Importantly, the Bensussan group had precisely localized the binding site on Eps15 for AP2 complexes by deletion analysis on the DIII domain of Eps15. They showed that a GST-protein containing the entire COOH-terminal domain of Eps15 could efficiently precipitate AP-2 complexes from cytosol. A construct lacking amino acids 661-739, GST-DIIIΔ1, could also precipitate AP-2 complexes albiet less efficiently. In contrast, GST-DIIIΔ2 a construct lacking amino acids 621-739, failed to precipitate AP-2. These data defined the AP-2 binding site on Eps15 and provided the basis for the design of dominant-negative and control constructs (i.e. GST-fusion proteins containing ,GST-DIII, or lacking, GST-DIIIΔ2, the Eps15 AP-2 binding site) for analysis of Eps15 function *in vivo* and *in vitro*.

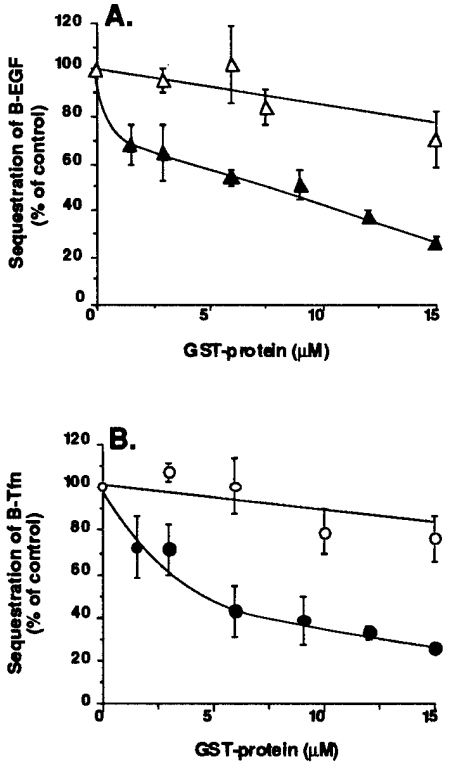

***Figure 1* GST-DIII inhibits both EGF and Tf endocytosis in perforated A431 cells.** The sequestration of B-EGF (A) or B-Tfn (B) were measured in perforated A431 cells in the presence of increasing concentrations of GST-DIII (closed symbols) or GST-DIIIΔ2 (open symbols).

To establish a role for Eps15 in receptor-mediated endocytosis we tested these fusion protein constructs in a cell free assay designed to study the early and late events of plasma membrane coated vesicle formation. This assay has been described extensively in previous reports of this grant. Briefly,

it makes use of human A431 cells that are perforated by mechanical disruption of the plasma membrane in order to wash out the endogenous cytosol. In the presence of purified cytosol and an ATP-regenerating system, biotinylated ligands (B-Tfn or B-EGF) are sequestered into deeply invaginated coated pits and become inaccessible to avidin (Schmid 1993). Since perforated A431 cells remain fully accessible to exogenous added reagents, Eps15-derived GST fusion proteins could be introduced in perforated A431 cells in order to test their effect on endocytosis. We reported last year that the recruitment of EGF-R into coated pits was inhibited *in vitro* in the presence GST-DIII. We have extended these results to show that that GST-DIII significantly inhibits not only B-EGF but also B-Tfn endocytosis. As seen in Figure 1 the inhibition of both EGF uptake (Panel a) and Tfn-uptake (Panel b) was dose-dependent and reached a plateau at 10 μM GST-DIII . In contrast, GST-DIIIΔ2, lacking the AP-2 binding site had no significant effect on either Tfn and EGF endocytosis. These results suggest that the inhibitory effect of DIII requires a functional AP-2 binding site. These data could be confirmed *in vivo* in intact cells by the Bensussan group. After transient transfection, HeLa cells overexpressing DIII and DIIIΔ2 fusion proteins tagged with GFP (Green Fluorescent Protein), did not show any endocytosis of Tfn conjugated to a fluorochrome.

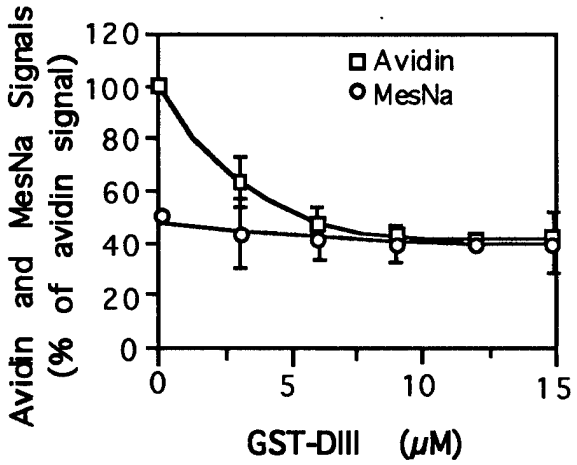

Figure 2 **GST-DIII selectively inhibits early events in endocytosis.** The sequestration of B-SS-Tfn into constricted coated pits and coated vesicles was detected by the inaccessibility to avidin (❑) whereas the sequestration of B-SS-Tf into coated vesicles only is measured by the resistance to MesNa (O). The data represent averages (±SD) of 3 experiments.

The inaccessibility of biotinylated Tfn to avidin, namely sequestration, can occur as a result of both its inclusion into constricted coated pits which remain plasma membrane associated and/or into coated vesicles, representing early and late events in coated vesicle formation respectively. The data presented in Figure 1b shows that the inhibition of the avidin signal by GST-DIII is not complete; thus GST-DIII could either partially inhibit both early and late events of coated vesicle formation, or inhibit solely early or late events. To discriminate between early and late events of clathrin coated vesicle formation, we used a modified version of the *in vitro* assay for transferrin endocytosis. The late events are selectively detected when Tfn, biotinylated through a cleavable disulphide bond (B-SS-Tfn), becomes inaccessible to the small membrane impermeant reducing agent, β-mercaptoethane sulfonate (MesNa) (Schmid 1993), The effect of the GST-DIII construct was studied within the same experiment, both on the avidin and the MesNa signals. As previously described (Carter et al. 1993; Lamaze et al. 1993), the MesNa signal in perforated A431 cells is about half of the avidin signal. This MesNa signal was not inhibited by GST-DIII at concentrations which inhibited the avidin signal (Fig 2). Importantly, at the inhibitory concentrations of GST-DIII, the avidin signal was superimposed with the MesNa signal. Altogether, the data obtained using the *in vitro*

assay strongly suggest that adding GST-DIII inhibits early, but not late events of coated vesicle formation.

The inhibitory effect of DIII observed on endocytosis could be the result of a competition between GST-DIII and endogenous Eps15 for the AP-2 binding site. To test this hypothesis we examined whether the DIII inhibitory effect could be prevented by an excess of purified AP-2 complexes. Thus, perforated A431 cells were incubated in the presence of purified AP-2 and the sequestration of B-EGF and B-Tfn were followed at inhibitory concentrations of GST-DIII. As shown in Figure 3, the addition of purified AP-2 complexes restored highly efficient endocytosis. Furhter confirmation of this hypothesis was obtained in another set of experiments performed by the Bensussan group. Cell lysates were incubated in the presence of GST fusion proteins prior to and during precipitation with the anti-Eps15 antibody, 6G4. As previously shown, 6G4 coimmunoprecipitated AP-2 with endogenous Eps15 from untreated lysates and from lysates treated with 10 µM GST. In contrast, the amount of AP-2 coimmunoprecipitated with endogenous Eps15 in lysates treated with 10 µM GST-DIII was markedly reduced (data not shown), indicating that GST-DIII competed with endogenous Eps15 for AP-2 binding. Altogether, our results indicate that the association of Eps15 with AP-2 is required for the efficient recruitment of Tfn and EGf receptors into coated pits.

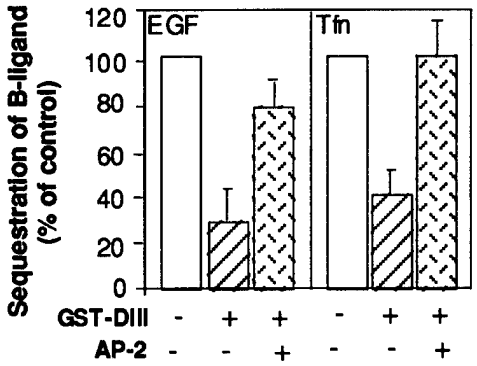

Figure 5: **GST-DIII competes with endogenous Eps15 for AP-2 binding** Sequestration assays for B-Tfn and B-EGF were performed with or without 12 µM GST-DIII and 0.3 mg/ml purified AP-2 as indicated.

CONCLUSION

During the past year have continued to explore the mechanisms regulating endocytosis of activated EGF-R, focusing more particularly on Eps15, an EGF-R tyrosine kinase substrate which induces a transformed phenotype when overexpressed in normal human fibroblasts (Fazioli et al., 1993). We have confirmed our initial findings that Eps15 was involved in the recruitment of activated EGF-R into coated pits. We have demonstrated that the binding of AP-2 to Eps15 is required for its function in endocytosis. These results will help to identify other partners of Eps15 and to understand the function of Eps15 in growth factor endocytosis, a key process in the control of breast tissue proliferation.

(8) REFERENCES

Benmerah, A., B. Bègue, A. Dautry-Varsat, N. Cerf-Bensussan (1996) The ear of α-adaptin interacts with the COOH terminal domain of the Eps15 protein. J. Biol. Chem. 271:12111-12116.

Benmerah, A., J. Gagnon, B. Bègue, B. Mégarbané, A. Dautry-Varsat and N. Cerf-Bensussan (1995) The tyrosine kinase substrate eps15 is constitutively associated with the plasma membrane adaptor AP-2 J. Cell Biol. 131:1831-1838

Carter, L. L., T. E. Redelmeier, et al. (1993). "Multiple GTP-binding proteins participate in clathrin-coated vesicle-mediated endocytosis." J. Cell Biol. 120: 37-45.

Fazioli, F., L. Minichiello, B. Matoskova, W. T. Wong and P. Paolo di Fiore (1993) eps15, a novel tyrosine kinase substrate, exhibits transfroming activity. Mol. Cell. Biol. 13:5814-5828

Kubler, E. and H. Riezman (1993). "Actin and fimbrin are required for the internalization step of endocytosis in yeast." EMBO J. 12: 2855-2862.

Lamaze, C. and Schmid, S.L. (1995) Recruitment of Epidermal Growth Factor Receptors into Coated Pits Requires Their Activated Tyrosine Kinase. J. Cell Biol. 129:47-54

Lamaze, C., Baba, T., Redelmeier, T.E. and S.L. Schmid (1993) Recruitment of EGF- and Transferrin Receptors into Coated Pits *in vitro*: Differing Biochemical Requirements. Mol. Biol. Cell 4: 715-727.

Lamaze, C., T. Baba, et al. (1993). "Recruitment of epidermal growth factor receptor and transferrin receptors into coated pits *in vitro*: differing biochemical requirements." Mol. Biol. Cell 3: 1181-1194.

Munn, A. L., B. J. Stevenson, et al. (1995). "end5, end6, and end7: mutations that cause actin delocalization and block the internalization step of endocytosis in Saccharomyces cerevisiae." Mol. Biol. Cell 6: 1721-1742.

Schmid, S. L. (1993). "Coated vesicle formation *in vitro*: conflicting results using different assays." Trends Cell Biol. 3: 145-148.

Schumacher, C., Knudsen, B. S., Ohuchi, T., Di Fiore, P. P., Glassman, R. H., and Hanafusa, H. (1995). The SH3 domain of Crk binds specifically to a conserved proline-rich motif in Eps15 and Eps15R. J. Biol. Chem. 270, 15341-7.

van Delft, S., Schumacher, C., Hage, W., Verkleij, A. J., and Henegouwen, P. (1997). Association and colocalization of Eps15 with adaptor protein-2 and clathrin. J. Cell Biol. 136, 811-821.

Vieira, A. V., Lamaze, C. and Schmid, S. L. (1996) "Control of EGF receptor signalling by clathrin-mediated endocytosis." Science 274: 2086-89.

Wells, A., Welsh, J. B., Lazar, C. S., Wiley, H. S. Gill, G. N., and Rosenfeld, M. G. (1990). Ligand-induced transformation by a non internalizing EGF receptor. Science 247: 962-964.

(9) BIBLIOGRAPHY:

Manuscripts resulting from this work (*included in Appendix)

Lamaze, C. and Schmid, S.L. (1995) Recruitment of Epidermal Growth Factor Receptors into Coated Pits Requires Their Activated Tyrosine Kinase. **J. Cell Biol.** 129:47-54

Lamaze, C. and S. L. Schmid (1995) The Emergence of Clathrin-Independent Pinocytic Pathways. **Curr. Opin. Cell Biol.**, 7: 573-580

*Lamaze, C., T.-H Chuang, L.J. Terlecky, G. M. Bokoch and S. L.Schmid (1996) Regulation of Transferrin Receptor-Mediated Endocytosis by Rho and Rac. **Nature** 382:177-179

*Vieira, A. V., C. Lamaze and S.L. Schmid (1996) Control of EGF Receptor Signalling by Clathrin-Mediated Endocytosis", **Science** 274:2086-2089

*Lamaze, C., L. M. Fujimoto, H.L Yin, and S.L. Schmid (1997) The actin cytoskeleton is required for receptor-mediated endocytosis in mammalian cells. **J. Biol. Chem.** 272:20332-20335

Benmerah, A., C. Lamaze, B. Bègue, S.L. Schmid, A. Dautry-Varssat and N. Cerf-Bensussan AP-2/Eps15 interaction is required for receptor-mediated endocytosis. *submitted to J. Cell Biol.*

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Regulation of receptor-mediated endocytosis by Rho and Rac

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Pinocytosis and membrane ruffling are among the earliest and most dramatic cellular responses to stimulation by growth factors or other mitogens[1]. The small Ras-related G proteins Rho and Rac have a regulatory role in membrane ruffling[1–3] and activated Rho has been shown to stimulate pinocytosis when microinjected into *Xenopus* oocytes[4]. In contrast to these well established effects of Rho and Rac on plasma membrane morphology and bulk pinocytosis, there has been no evidence for their involvement in the regulation of receptor-mediated endocytosis in clathrin-coated pits. Here we show that activated Rho and Rac inhibit transferrin-receptor-mediated endocytosis when expressed in intact cells. Furthermore, we have reconstituted these effects in a cell-free system and established that Rho and Rac can regulate clathrin-coated vesicle formation.

Receptor-mediated endocytosis of Texas red-conjugated transferrin was measured in transiently transfected cells expressing wild-type or mutant forms of Rac and Rho. Intracellular accumulation of transferrin was potently (>95% of transfected cells) inhibited in HeLa cells transiently expressing an activated, GTP-bound Rac mutant (Q61L), in which a glutamine residue is substituted for a leucine at position 61 (Fig. 1*e*,*f*), but not affected (<5%) in cells expressing either wild-type Rac (Fig. 1*a*, *b*) or an inactivated, GDP-bound mutant, Rac^{T17N}(asparagine residue substituted for threonine at position 17; Fig. 1*c*, *d*). Similar results were obtained with activated Rho^{Q63L} (leucine instead of glutamine at position 63; Fig. 1*g*, *h*), but not with either a GDP-Rho mutant (T19N) or wild-type Rho (data not shown).

Inhibition of intracellular transferrin accumulation in transiently transfected cells could reflect Rac and Rho effects at any stage of the internalization, sorting and recycling pathway taken by transferrin receptors. Although we cannot rule out effects of Rho-family GTPases on subsequent endocytic membrane trafficking, their role in internalization was further supported by our finding that cells expressing the activating mutant of Rac (Q61L), but not wild-type or Rac^{T17N}, could not be infected with vesicular stomatitis virus (data not shown). Nevertheless, to confirm and investigate further their early role in endocytosis, we tested the effects of Rho and Rac in a cell-free system that reconstitutes the internalization of receptor-bound transferrin into clathrin-coated pits[5,6]. Rac and Rho were both potent inhibitors (half-maximal inhibition at ~25–30 nM) of transferrin-receptor-mediated endocytosis in perforated A431 cells (Fig. 2*a*). The inhibitory effects of Rac and Rho must require that they be post-translationally

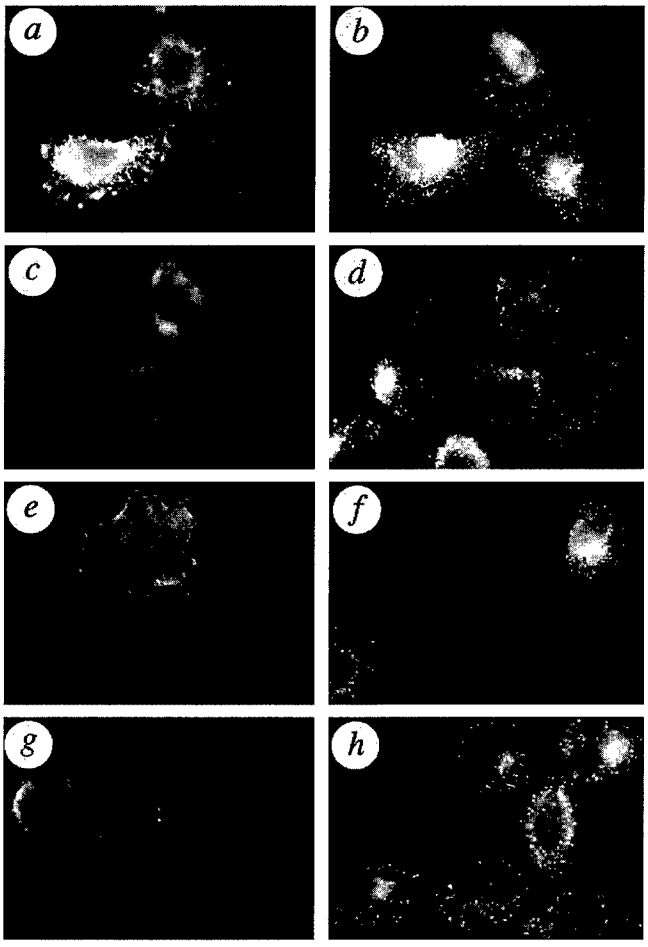

FIG. 1 Intracellular accumulation of transferrin (Tfn) is blocked in transiently transfected cells expressing activated Rac and Rho mutants. HeLa cells were grown on coverslips and transfected with expression plasmids encoding Flag-tagged wild-type Rac (*a*, *b*), Rac^{T17N} (*c*, *d*), Rac^{Q61L} (*e*, *f*) or haemagglutinin (HA)-tagged Rho^{Q63L} (*g*, *h*) as described[27]. 24 h after transfection, cells were incubated with Texas-red-conjugated Tfn (Molecular Probes) for 15 min at 37 °C, washed and fixed with 4% paraformaldehyde (PFA) for indirect immunofluorescence as described[28]. Internalized Tfn accumulated in punctate endosomal structures within the cytoplasm (*b*, *d*, f and *h*). Cells expressing Rac or Rho molecules were detected by co-immunostaining with anti-Flag (*a*, *c*, *e*) or anti-HA antibodies, respectively.

processed because proteins expressed in *Escherichia coli* had no effect (data not shown).

Inhibition by wild-type GTPases *in vitro* could reflect quantitative differences in the amount of protein assayed or differences in the activity of GTPase-activating protein (GAP) *in vivo* and *in vitro*. We were also unable to discern differences in the ability of Rac or Rho to inhibit endocytosis *in vitro* when preloaded either with GDP-βS or GTP-γS, presumably because these GTPases are able rapidly to exchange nucleotides in cell lysates[7]. Therefore, to confirm the specificity of inhibition by activated forms of Rho and Rac as seen *in vivo*, assays were done in the presence of excess rhoGDI, a protein that forms a stoichiometric complex with all members of the Rho GTPase family, inhibits GDP–GTP exchange and locks them in their inactivated GDP-bound states (hence GDI, GDP-dissociation inhibitor)[8]. Inclusion of rhoGDI in the assay substantially blocked the inhibitory effects of Rho and Rac (Fig. 2*b*), indicating that the GTP-bound forms of Rho and Rac inhibit an early event in receptor-mediated endocytosis both *in vivo* and *in vitro*.

To determine whether Rho or Rac activities are constitutively required for receptor-mediated endocytosis, perforated A431 cells were incubated in the presence of rhoGDI alone. Addition of either rhoGDI or its homologue Ly-GDI/D4 (refs 9,10) stimulated receptor-mediated endocytosis of transferrin (Fig. 2*c*). This result argues that Rho and Rac are not required for endocytosis and instead indicates that endogenous Rho and Rac might be negative regulators of endocytosis under the conditions of our assay. Consistent with this, recombinant C3 transferase, a bacterial toxin that specifically inactivates Rho through ADP-ribosylation of Asn 41 (ref. 11) also stimulated transferrin receptor-mediated endocytosis in perforated A431 cells (Fig. 2*d*). Thus, activation and inactivation of Rho have opposing effects on transferrin sequestration *in vitro*. In all cases, receptor-mediated endocytosis of epidermal growth factor (EGF) was similarly affected (data not shown), indicating that Rho and Rac are general regulators of endocytosis via clathrin-coated vesicles. It should be noted that the effects seen with Rho-family GTPases were selective, because Rab-family GTPases, rabGDI or Arf1 GTPase did not have any effect on endocytosis *in vitro* (L.J.T. and S.L.S., unpublished observations).

In some cases, Rac can act through Rho in regulating downstream events[1,12]. Thus, the Rac effects could either be mediated directly through a common downstream effector of both GTPases or, indirectly, through Rho. However, we found that C3 transferase, which specifically inactivates endogenous Rho, could not block inhibition by Rac (Fig. 2*e*). Thus, Rac inhibition appears to be independent of Rho.

The overall process of coated vesicle formation *in vitro* can be dissected into early events, leading to the sequestration of receptor-bound transferrin into constricted coated pits, and late events involved in coated vesicle budding[6]. Coated vesicle budding is selectively detected when transferrin, biotinylated through a cleavable disulphide bond, is internalized into sealed membrane vesicles and becomes inaccessible to the small membrane impermeant reducing agent, β-mercaptoethane sulphonic acid (MesNa). Using this assay, we found that both the rate and

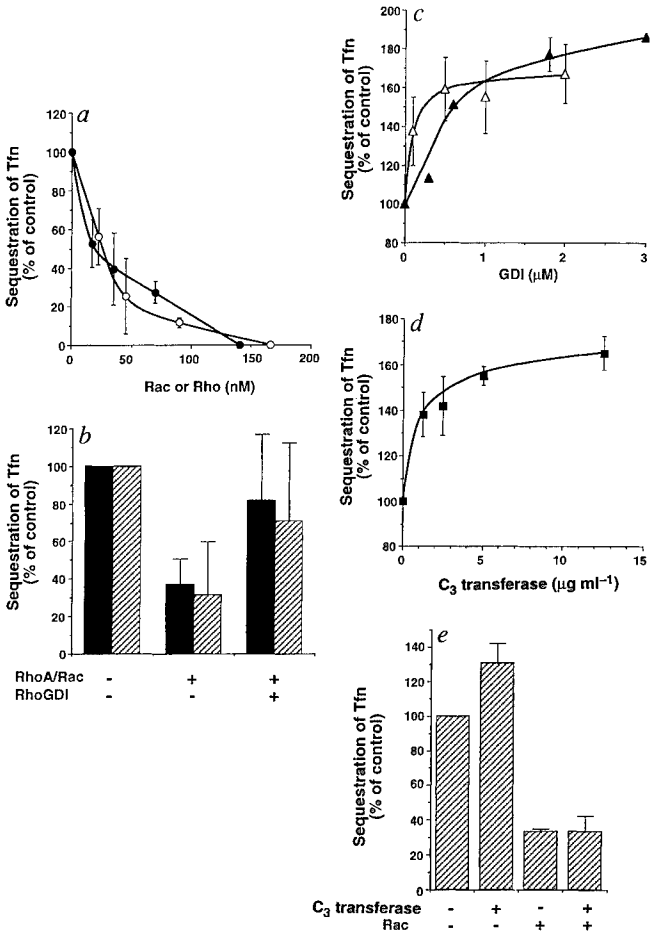

FIG. 2 Regulation by Rac and Rho of Tfn-receptor-mediated endocytosis in a cell-free system. Perforated A431 cells were incubated for 30 min at 37 °C with K562 erythroleukaemic cell cytosol, an ATP-regenerating system and biotinylated Tfn (B-Tfn). Incubations were stopped on ice and Tfn-receptor-mediated endocytosis determined by measuring the sequestration of B-Tfn from exogenously added avidin as described[5]. *a*, Assays were done in the presence of increasing concentrations of wild-type Rac (open circles) or Rho (filled circles) prepared from baculovirus-infected Sf9 cells as described[29]. The efficiency of cytosol- and ATP-dependent Tfn sequestration in control incubations done in the absence of added Rho and Rac was 35 ± 8% of total cell-associated Tfn. Data shown are the average ±s.d. from 5 independent experiments. *b*, Assay in the presence or absence of 45 nM Rac (hatched bars) or 35 nM Rho (solid bars) with or without 1 μM rhoGDI, prepared as described[30]. *c*, Assay in the presence of increasing concentrations of purified recombinant rhoGDI (△) or Ly-GDI/D4 (▲), or *d*, in the presence of a fusion protein of glutathione-S-transferase (GST) with recombinant C3 transferase (from S. Dillon, Tufts University) expressed and purified from *E. coli*. *e*, Assay with or without GST–C3 transferase (5 μg ml^{-1}) and/or Rac (45 nM), as indicated. Data shown are the average ±s.d. from 3 independent experiments.

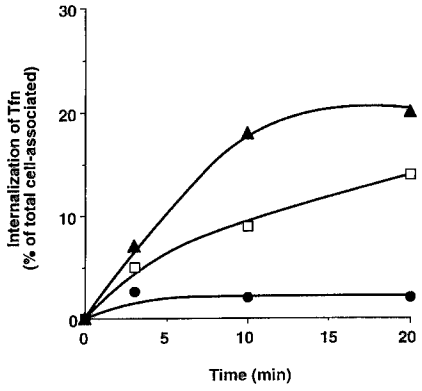

FIG. 3 Rho and rhoGDI affect clathrin-coated vesicle budding in a cell-free system. Perforated A431 cells were incubated with Tfn which had been biotinylated at a cleavable disulphide bond (BSS-Tfn) under control conditions (squares) in the presence of cytosol and ATP, or in the presence of 35 nM Rho (circles) or 1 μM rhoGDI (triangles). Incubations were stopped on ice and the internalization of receptor-bound Tfn into sealed coated vesicles was determined by measuring the resistance of BSS-Tfn to cleavage by the small membrane-impermeant reducing agent MesNa, as described[5]. The results show ATP- and cytosol-dependent signals obtained after subtraction of background from cells incubated at 37 °C for 30 min in the presence of an ATP-depleting system and in the absence of cytosol.

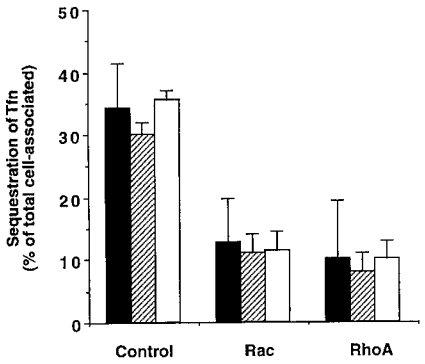

FIG. 4 Inhibition of endocytosis by Rac or Rho is not affected by either cytochalasin D or phalloidin. Tfn sequestration was assayed in a cell-free system as for Fig. 2, in the absence (black bars) or presence of either 10 μM phalloidin (white bars) or 10 μg ml^{-1} cytochalasin D (hatched bars) without (control) or with either Rac (45 nM) or Rho (35 nM), as indicated. Control experiments, both *in vivo* and *in vitro*, in which cells were fixed, permeabilized and labelled with Texas-red–phalloidin confirmed that cytochalasin D effectively disrupted actin stress fibre formation in the presence or absence of Rho and Rac (data not shown, but see ref. 12). Data are expressed as the percentage of total cell-associated ligand inaccessible to avidin after subtraction of background, as described for Fig. 3.

extent of coated vesicle budding were severely inhibited in the presence of Rho (or Rac; data not shown) and, in a reciprocal manner, were enhanced by addition of rhoGDI (Fig. 3).

Our data indicate that the effects of Rho and Rac could be mediated by a common downstream effector. As both of these small GTPases control, directly or indirectly, assembly of the actin cytoskeleton[2,3], we investigated whether their effects on endocytosis might be related to effects on actin by testing whether cytochalasin D, which inhibits actin assembly, or phalloidin, which stabilizes actin filaments, would antagonize or synergize with Rac and Rho. As shown in Fig. 4, neither cytochalasin D (at 1–10 μg ml^{-1}) nor phalloidin (at 0.1–10 μM) inhibit receptor-mediated endocytosis of transferrin on their own. More important, neither altered the inhibitory effects of Rac and Rho on transferrin endocytosis. Cytochalasin D treatment likewise did not affect the inhibition of transferrin accumulation seen in cells transiently transfected with either mutant Rac or Rho (data not shown). These results argue against the simple hypothesis that Rho and Rac effects are due to triggered assembly of the actin cytoskeleton. However, the effects of cytochalasin D on cortical actin assembly/disassembly are poorly characterized and we therefore cannot rule out a role for this subpopulation of actin filaments in regulating endocytosis.

The common downstream effector(s) responsible for the regulation of clathrin-mediated endocytosis by Rho and Rac have not been identified. Possible candidates include phospholipase D (PLD) (refs 13,14), phosphoinositol-4-phosphate 5-OH kinase (PI(5)K) (refs 15–17) and PI(3)K (refs 17,18) which has also been identified as an upstream regulator of Rac and is required for growth-factor-stimulated membrane ruffling[19–21]. However, wortmannin, a potent inhibitor of PI(3)K, has no effect either on transferrin- or EGF endocytosis in intact or perforated cells (refs 21,22; C.L. and S.L.S., unpublished observations). It remains possible that activation of PLD and/or PI(5)K might directly control clathrin-coated vesicle formation, consistent with recent proposals that site-directed lipid modifications could trigger the recruitment of components required to initiate vesicle budding[23,24]. Alternatively, activated Rho or Rac could indirectly affect coated-vesicle formation by mistargeting, to multiple intracellular sites, the assembly of limiting components essential for coated-vesicle budding. In this context, it is interesting that GTP-γS treatment of cells results in the mistargeting of AP2 adaptor complexes from coated pits on the plasma membrane to the endosomal compartment[25]. Receptor-mediated endocytosis *in vitro*, only the second cell-free assay system in which the effects of Rho and Rac have been reconstituted, should provide a functional means for identifying the relevant downstream effectors.

In summary, our results are strong evidence for a previously unsuspected role for Rho and Rac in the regulation of receptor-mediated endocytosis through coated pits. This is in marked contrast to the stimulatory effects of Rac and Rho on fluid-phase endocytosis in mammalian cells[1] and *Xenopus* oocytes[4], respectively. Our findings provide further support for the mechanistic distinction between clathrin-mediated endocytosis and clathrin-independent pinocytic events[26]. □

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1. Ridley, A. J. & Hall, A. *Cell* **70,** 401–410 (1992).
2. Hall, A. *A. Rev. Cell Biol.* **10,** 31–54 (1994).
3. Takai, Y., Sasaki, T., Tanake, K. & Nakanishi, H. *Trends. biochem. Sci.* **20,** 227–231 (1995).
4. Schmalzing, G. *et al. J. Cell Biol.* **130,** 1319–1332 (1995).
5. Smythe, E., Redelmeier, T. E. & Schmid, S. L. *Meth. Enzym.* **219,** 223–234 (1992).
6. Schmid, S. L. *Trends Cell Biol.* **3,** 145–148 (1993).
7. Bokoch, G. M., Boh., B. P. & Chuang, T.-H. *J. biol. Chem.* **269,** 31674–31679 (1994).
8. Ueda, T., Kikuchi, A., Ohgo, N., Yamamoto, J. & Takai, Y. *J. biol. Chem.* **265,** 9373–9380 (1990).
9. Lelias, J. M. *et al. Proc. natn. Acad. Sci. U.S.A.* **90,** 1479–1483 (1993).
10. Scherle, P., Behrens, T. & Staudt, L. M. *Proc. natn. Acad. Sci. U.S.A.* **90,** 7568–7572 (1989).
11. Aktories, K. & Hall, A. *Trends pharmac. Sci.* **10,** 415–418 (1989).
12. Nobes, C. D. & Hall, A. *Cell* **81,** 53–62 (1995).
13. Malcolm, K. C., Ross, A. H., Qui, R-G., Symons, M. & Exton, J. H. *J. biol. Chem.* **269,** 25951–25954 (1994).
14. Singer, W. D., Brown, H. A., Bokoch, G. M. & Sternweis, P. C. *J. biol. Chem.* **270,** 14944–14950 (1995).
15. Chong, L. D., Traynor-Kaplan, A., Bokoch, G. M. & Schwartz, M. A. *Cell* **79,** 507–513 (1994).
16. Hartwig, J. H. *et al. Cell* **82,** 643–653 (1995).
17. Tolias, K. F., Cantley, L. C. & Carpenter, C. L. *J. biol. Chem.* **270,** 17656–17659 (1995).
18. Bokoch, G. M., Vlattos, C. J., Wang, Y., Knaus, V. G. & Traynor-Kaplan, A. E. *Biochem. J.* **315,** 775–779 (1996).
19. Kotani, K. *et al. EMBO J.* **13,** 2313–2321 (1994).
20. Wennström, S. *et al. Curr. Biol.* **4,** 385–393 (1994).
21. Claque, M. J., Thorpe, C. & Jones, A. T. *FEBS Lett.* **367,** 272–274 (1995).
22. Lamaze, C. & Schmid, S. L. *J. Cell Biol.* **129,** 47–54 (1995).
23. Liscovitch, M. & Cantley, L. C. *Cell* **81,** 659–662 (1995).
24. DeCamilli, P., Emr, S. D., McPherson, P. S. & Novick, P. *Science* **271,** 1533–1539 (1996).
25. Seaman, M. N. J., Ball, C. L. & Robinson, M. S. *J. Cell Biol.* **123,** 1093–1105 (1993).
26. Lamaze, C. & Schmid, S. L. *Curr. Opin. Cell Biol.* **7,** 573–580 (1995).
27. Zhang, S. *et al. J. biol. Chem.* **270,** 23934–23936 (1995).
28. Damke, H., Baba, T., van der Bliek, A. M. & Schmid, S. L. *J. Cell Biol* **131,** 69–80 (1995).
29. Xu, X., Barry, D. C., Settleman, J., Schwartz, M. A. & Bokoch, G. M. *J. biol. Chem.* **269,** 23569–23574 (1994).
30. Chuang, T.-H., Xu, X., Knaus, U. G., Hart, M. J. & Bokoch, G. M. *J. biol. Chem.* **268,** 775–778 (1993).

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Control of EGF Receptor Signaling by Clathrin-Mediated Endocytosis

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Control of EGF Receptor Signaling by Clathrin-Mediated Endocytosis

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Epidermal growth factor receptor (EGFR) signaling was analyzed in mammalian cells conditionally defective for receptor-mediated endocytosis. EGF-dependent cell proliferation was enhanced in endocytosis-defective cells. However, early EGF-dependent signaling events were not uniformly up-regulated. A subset of signal transducers required the normal endocytic trafficking of EGFR for full activation. Thus, endocytic trafficking of activated EGFR plays a critical role not only in attenuating EGFR signaling but also in establishing and controlling specific signaling pathways.

Signaling by ligand-activated receptor tyrosine kinases (RTKs) such as EGFR can elicit a wide range of cell type–specific responses leading to proliferation or differentiation. EGF binding triggers dimerization and trans- or autophosphorylation of the receptor, followed by recruitment and activation of SH2 (src homology domain 2) or PTB (phosphotyrosine binding) domain–containing intracellular signal transducers (*1*). Ligand binding also triggers the recruitment of EGFR to clathrin-coated pits, followed by internalization of the EGFR-ligand complex and its delivery to lysosomes for degradation (*2*). Although activated EGFR follows the canonical endocytic pathway (*3*) during this process of "down-regulation," RTK-specific regulators affecting sorting at both early (*4*, *5*) and late (*6*, *7*) trafficking steps have been identified. Many of these regulators, for example, snx-1 (sorting nexin 1) (*6*) and phosphatidylinositol 3-kinase (PI-3-kinase) (*7*), appear to affect sorting of only a subset of RTKs. Why should RTKs have their own repertoire of intracellular trafficking regulators? One possibility is that regulation of trafficking serves to modulate RTK signaling.

To examine whether EGFR endocytosis and trafficking are important for controlling the signaling pathways and cellular responses to EGF, we examined these events in cells conditionally and specifically defective in clathrin-dependent receptor-mediated endocytosis (*8*). The conditional defect in endocytosis is imposed by the regulated expression of the $Lys^{44} \rightarrow Ala^{44}$ (K44A) mutant form of dynamin (*8*), a guanosine triphosphatase that is required for clathrin-coated vesicle formation (*9*).

Ligand-induced endocytosis of EGFR was potently inhibited in cells overexpressing K44A dynamin (K44A cells) as compared with cells overexpressing comparable amounts of wild-type dynamin (WT cells) (Fig. 1A). In contrast, endocytosis of inactivated EGFR, defined as the basal rate and measured with mAb528 [an antagonistic monoclonal antibody (mAb) to EGFR] as ligand (*10*), was not significantly affected (Fig. 1A). These data confirm the role of

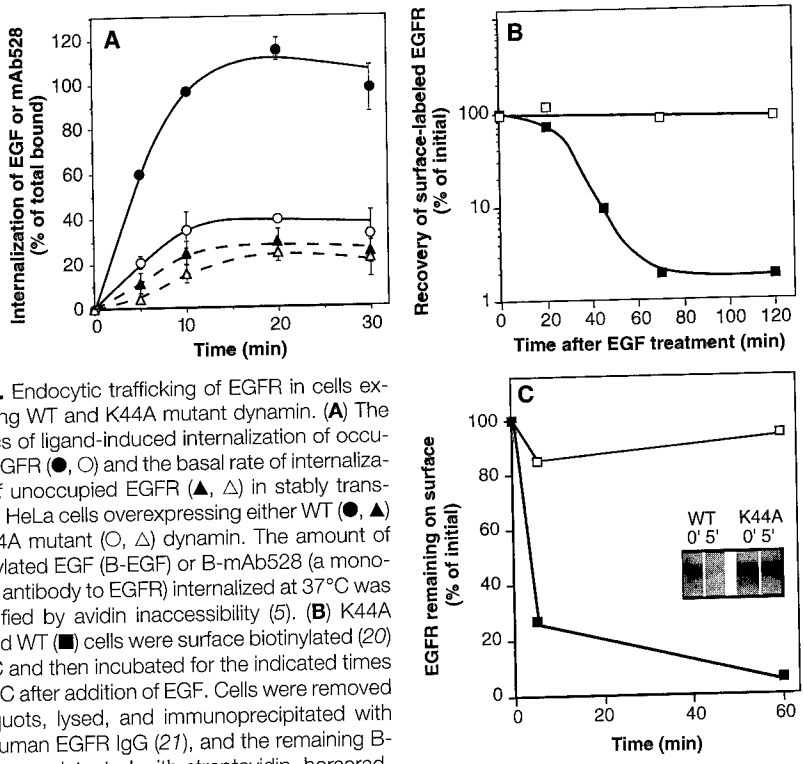

Fig. 1. Endocytic trafficking of EGFR in cells expressing WT and K44A mutant dynamin. (**A**) The kinetics of ligand-induced internalization of occupied EGFR (●, ○) and the basal rate of internalization of unoccupied EGFR (▲, △) in stably transfected HeLa cells overexpressing either WT (●, ▲) or K44A mutant (○, △) dynamin. The amount of biotinylated EGF (B-EGF) or B-mAb528 (a monoclonal antibody to EGFR) internalized at 37°C was quantified by avidin inaccessibility (*5*). (**B**) K44A (□) and WT (■) cells were surface biotinylated (*20*) at 4°C and then incubated for the indicated times at 37°C after addition of EGF. Cells were removed in aliquots, lysed, and immunoprecipitated with anti-human EGFR IgG (*21*), and the remaining B-EGFR was detected with streptavidin–horseradish peroxidase. (**C**) K44A (□) and WT (■) cells were treated with EGF for the indicated times, then surface biotinylated at 4°C (*20*) and analyzed for B-EGFR after immunoprecipitation (*21*).

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clathrin-coated pits in ligand-induced EGFR endocytosis and suggest that unoccupied receptors are internalized along with the bulk plasma membrane (*11*).

Intracellular accumulation of EGF in K44A cells (Fig. 1A) reached a steady state by 10 min, which suggests that intracellular ligand-receptor complexes are either rapidly degraded or recycled. To distinguish these two possibilities, we compared EGFR down-regulation and the surface expression of EGFR in WT and K44A cells. In the absence of EGF, both WT and K44A cells expressed equal levels of the endogenous EGFR (Fig. 1, B and C). In WT cells, after a brief lag, surface EGFR was degraded with a half-time of ~30 min, whereas in K44A cells ~80% of EGFR was detected even 2 hours after EGF addition (Fig. 1B). Correspondingly, EGFR was rapidly and efficiently cleared from the surface of WT cells but not K44A cells after addition of EGF (Fig. 1C). Thus, after internalization at a basal rate, activated EGFR appeared to be efficiently recycled back to the cell surface in K44A cells, which is consistent with a previous model suggesting that endocytosis rates, not EGFR kinase activation, control EGFR down-regulation (*10*). In the absence of EGF, neither K44A nor WT cells showed any significant loss of EGFR over the 2-hour time course (*12*). Thus, in K44A cells EGFR is not subject to normal ligand-induced intracellular trafficking and degradation.

Responses to EGF were then analyzed in cells with WT or defective EGFR trafficking. First, as an endpoint of a complex series of signal transduction events, EGF-dependent proliferation of K44A and WT cells was measured after serum starvation. K44A cells showed increased EGF-dependent proliferation as assessed by either cell number–dependent catabolism of the dye MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] or [^{3}H]thymidine incorporation into DNA (Fig. 2, A and B). Furthermore, when K44A cells were grown in the presence of tetracycline to suppress mutant dynamin expression, they behaved indistinguishably from WT cells grown with or without induction of dynamin. Thus, overexpression of K44A dynamin, but not WT dynamin, enhanced the cell proliferative response. Because the overexpression of WT or K44A dynamin does not alter the levels of endogenous EGFR, these results establish that defective endocytic trafficking of EGFR leads to enhanced EGF-dependent proliferation, which confirms that receptor-mediated endocytosis is critical for attenuating EGFR signaling (*13*).

Early responses to EGF were examined by analyzing EGF-dependent tyrosine phosphorylation in WT and K44A cells. After serum starvation, both WT and K44A cells showed significant EGF-dependent increases in tyrosine phosphorylation, which indicates that overexpression of neither WT nor K44A dynamin led, in general, to EGF-independent activation of signaling pathways (Fig. 2C). In contrast, several proteins were either hyperphosphorylated or hypophosphorylated in K44A cells relative to WT cells after EGF stimulation (Fig. 2C). Thus, normal EGFR endocytic trafficking was required to trigger distinct signaling pathways.

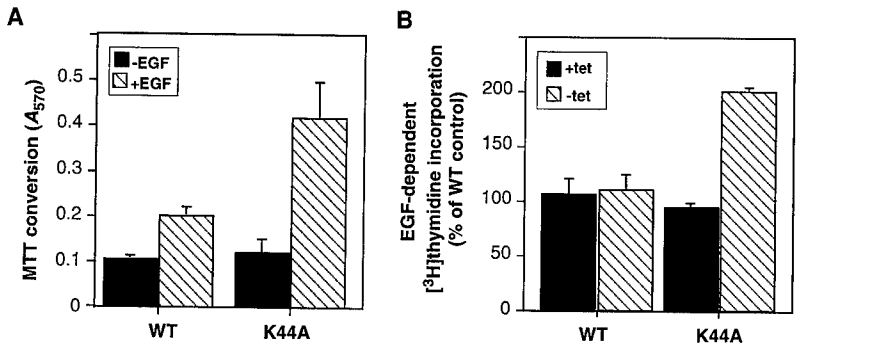
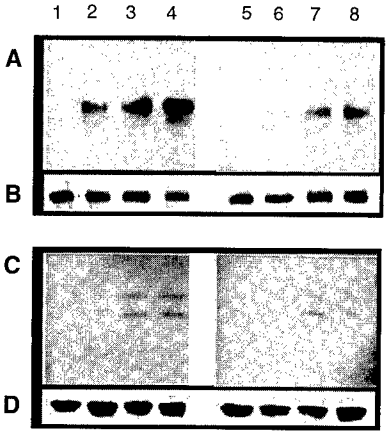


Fig. 2. EGF-dependent proliferation and tyrosine phosphorylation patterns in WT and K44A cells. (**A**) Serum-starved HeLa cells overexpressing either WT or K44A dynamin were treated with (striped bars) or without (solid bars) 4 nM EGF. After 48 hours, the enzymatic conversion of MTT was measured by absorbance at 570 nm (A_{570}) (*22*). SD (n = 3) is indicated above the bars. (**B**) K44A or WT cells were incubated in serum-free media in the presence (uninduced, solid bars) or absence (induced, shaded bars) of tetracycline (tet; 1 μg/ml). EGF-dependent incorporation of [^{3}H]thymidine into DNA was then measured (*22*) as an indication of mitogenesis. The data are shown relative to labeled DNA in cells not treated with EGF (± SD, n = 4). (**C**) HeLa cells overexpressing WT or K44A dynamin were treated (+) or not (−) with 3.5 nM EGF for 10 min. Tyrosine-phosphorylated proteins were immunoprecipitated (*21*) from whole-cell extracts with α-PY IgG (4G10) and then protein immunoblotted with the same antibody. Molecular mass standards are indicated on the left; components hyper- and hypophosphorylated in K44A relative to WT cells are indicated on the right by closed and open circles, respectively.



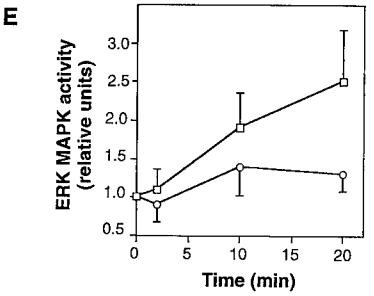


Fig. 3. EGF-dependent tyrosine phosphorylation of EGFR and MAPK (ERK1 and ERK2) is suppressed in endocytosis-defective cells. (**A** through **D**) Serum-starved K44A cells cultured in the presence (lanes 1 through 4) or absence (lanes 5 through 8) of tetracycline were treated with EGF for 0 min (lanes 1 and 5), 2 min (lanes 2 and 6), 10 min (lanes 3 and 7), or 20 min (lanes 4 and 8). Lysates prepared from equal numbers of cells were immunoprecipitated (*21*) with rabbit anti-human EGFR [(A) and (B)] or rabbit anti-rat ERK IgG [(C) and (D); upper and lower signals in (C) represent ERK1 and ERK2, respectively] and then immunoblotted with anti-phosphotyrosine [(A) and (C)], anti-EGFR (B), or anti ERK1 (D). (**E**) MAPK activity was assessed by myelin basic protein phosphorylation (*23*) with the use of anti-ERK immunoprecipitates of lysates from serum-starved WT (squares) or K44A (circles) HeLa cells that had been treated with EGF for the indicated times. [^{32}P]MBP was quantitated by densitometric scanning of autoradiographs (± SD, n = 3). MAPK activity in EGF-treated cells was expressed relative to that in untreated control cells.

To identify these pathways, the activation of known EGFR signal transducers was analyzed. EGFR was tyrosine-phosphorylated to a lesser extent in cells that overexpressed K44A dynamin (Fig. 3A) than in either WT cells (Fig. 4F) or in noninduced K44A cells (Fig. 3A). These differences were even more pronounced 20 min after addition of EGF, because EGFR degradation would have begun in WT cells (Fig. 3B). Thus, normal endocytic trafficking of activated EGFR was necessary to achieve full EGFR tyrosine phosphorylation. As is consistent with this observation, EGFR is hyperphosphorylated in endosomal versus plasma membrane fractions from rat liver (*14*). Furthermore, we have confirmed that hypophosphorylated EGFR can trigger enhanced proliferation (*15*).

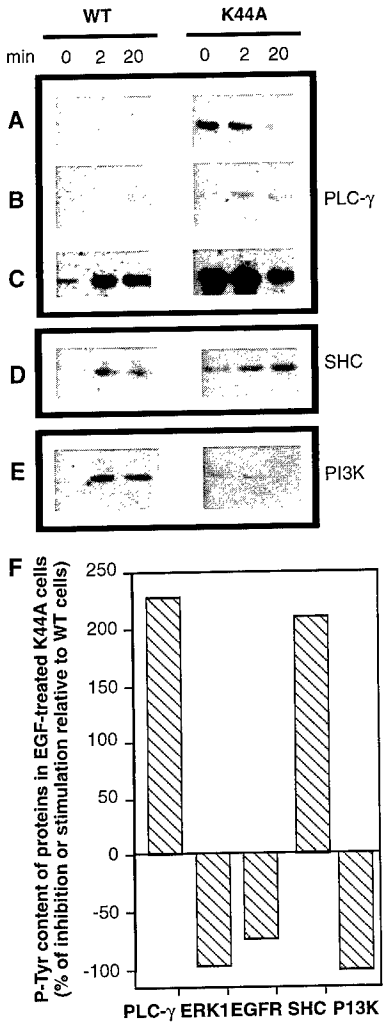


Fig. 4. Differential tyrosine phosphorylation of signal transduction components in endocytosis-defective cells. (**A** through **E**) EGF treatment of serum-starved WT or K44A cells for the indicated times was followed by immunoprecipitation of the extracts (*20*) with anti-phosphotyrosine IgG and then protein immunoblotting with mAb to bovine phospholipase Cγ–1 (PLC-γ) [(A) through (C)]; antibody to human SHC [SH2-domain containing α2 collagen-related protein; intermediate (p52/p55) form shown] (D); or antibody to rat p85 (PI3K), the 85-kD regulatory subunit of PI3K (E). The cells in (B) were cultured in the presence of tetracycline to provide an uninduced control. For comparison, in both (A) and (B) the same exposure time was used and (C) represents a longer exposure of (A). Control blots showed that the levels of SHC, PLC-γ, ERK1, and PI3K-p85 in WT and K44A cell lysates were similar (100 ± 16%) at all time points. (**F**) A quantitation of antiphosphotyrosine (anti–P-Tyr) protein immunoblot signals (20 min after EGF treatment) for the indicated signal transducers in HeLa cells overexpressing either WT or K44A dynamin. Each value represents an average of two experiments (average variation 29% for SHC, <12% for all others).

Activation of mitogen-activated protein (MAP) kinases was also suppressed in endocytosis-defective cells, as measured by both tyrosine phosphorylation of the MAP kinases ERK1 and ERK2 (Figs. 3C and 4F) and by MAP kinase activity (Fig. 3E). Thus, normal endocytic trafficking of EGFR is important for the full activation of MAP kinases (*16*).

The 85-kD regulatory subunit of PI 3-kinase (p85-PI3K) can be tyrosine-phosphorylated in response to EGF (*17*), although p85 tyrosine phosphorylation does not necessarily correlate with activation of the PI3K catalytic subunit. We found p85 to be hypophosphorylated in endocytosis-defective HeLa cells relative to WT cells in response to EGF (Fig. 4, E and F). Because PI3K activity is required for regulating receptor trafficking in endosomal compartments but not for internalization (*7*), our findings may reflect preferential activation of p85-PI3K during later events in intracellular trafficking of EGFR. In any case, these results provide an additional example of the requirement for correct EGFR trafficking in establishing specific signaling pathways.

Among the signaling proteins that were hyperphosphorylated in endocytosis-defective cells were PLC-γ (Fig. 4, A through C and F) and SHC (Fig. 4, D and F). PLC-γ showed increased phosphorylation even in the absence of EGF, although there was not a general up-regulation of EGF-independent tyrosine phosphorylation (Fig. 2C). The significance of this finding remains to be determined. The hyperphosphorylation of PLC-γ and SHC or both (*15*) may be an important factor in the enhanced EGF-dependent hyperproliferative pathway of these cells.

Thus, in addition to initiating the clearance and down-regulation of the EGF-EGFR signaling complex, receptor trafficking plays a critical role in defining the signals transmitted by activated EGFR. Our results provide functional insight into the physiological significance of RTK-specific regulators of receptor trafficking. A comparison of the mitogenic potencies of EGF (which diverts internalized receptor-ligand complexes to late endosomes and lysosomes) and transforming growth factor–α (which dissociates from EGFR at mild acidic pH, allowing it to recycle from early endosomes) suggests that EGFR ligands have evolved to regulate their signals by controlling EGFR trafficking (*18*). Signal transducing molecules have been shown to affect membrane trafficking (*19*); here we present evidence that this regulation can flow in both directions, that is, that membrane trafficking can also regulate signal transduction events.

REFERENCES AND NOTES

1. Reviewed by P. van der Geer, T. Hunter, R. A. Lindberg, *Annu. Rev. Cell Biol.* **10**, 251 (1994); J. Schlessinger and A. Ullrich, *Neuron* **9**, 383 (1992).
2. H. T. Haigler, J. M. McKanna, S. Cohen, *J. Cell Biol.* **81**, 382 (1979).
3. A. Sorkin and C. M. Waters, *Bioessays* **15**, 375 (1993).
4. A. Sorkin and G. Carpenter, *Science* **261**, 612 (1993); A. Benmerak *et al.*, *J. Cell Biol.* **131**, 1831 (1995); Z. Wang and M. F. Moran, *Science* **272**, 1935 (1996).
5. C. Lamaze, T. Baba, T. E. Redelmeier, S. L. Schmid, *Mol. Biol. Cell* **4**, 715 (1993); C. Lamaze and S. L. Schmid, *J. Cell Biol.* **129**, 47 (1995).
6. R. C. Kurten, D. L. Cadena, G. N. Gill, *Science* **272**, 1008 (1996).
7. M. Joly, A. Kazlauskas, R. S. Fay, S. Corvera, *ibid.* **263**, 684 (1994); H. Schpetner, M. Joly, D. Hartley, S. Corvera, *J. Cell Biol.* **132**, 595 (1996).
8. H. Damke, T. Baba, D. E. Warnock, S. L. Schmid, *J. Cell Biol.* **127**, 915 (1994).
9. J.-P. Liu and P. J. Robinson, *Endocr. Rev.* **16**, 590 (1995); D. E. Warnock and S. L. Schmid, *Bioessays* **18**, 885 (1996).
10. H. S. Wiley *et al.*, *J. Biol. Chem.* **266**, 11083 (1991).
11. Endocytosis rates for bulk membrane are maintained in cells overexpressing dominant-negative mutants of dynamin by the compensatory up-regulation of clathrin-independent endocytic mechanisms [H. Damke, T. Baba, A. M. van der Bliek, S. L. Schmid, *J. Cell Biol.* **131**, 69 (1995); C. Lamaze and S. L. Schmid, *Curr. Opin. Cell Biol.* **7**, 5730 (1995)].
12. A. Vieira and S. L. Schmid, unpublished data.
13. Cells that overexpress truncated EGFR deficient in ligand-induced endocytosis exhibit increased EGF-dependent proliferation [A. Wells *et al.*, *Science* **247**, 962 (1990); H. Masui, A. Wells, C. S. Lazar, M. G. Rosenfeld, G. N. Gill, *Cancer Res.* **51**, 6170 (1991)]. In these cases, however, the proliferative response may be due to altered signaling that results from abnormal receptor structure (and overexpression, or both) and not specifically to the lack of endocytosis [S. J. Decker, C. Alexander, T. Habib, *J. Biol. Chem.* **267**, 1104 (1992)].
14. G. M. DiGuglielmo, P. C. Baass, W.-J. Ou, B. I. Posner, J. J. M. Bergeron, *EMBO J.* **13**, 4269 (1994).
15. SHC tyrosine phosphorylation is an important requirement for enhanced proliferation mediated by hypophosphorylated EGFR [N. Gotoh *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 167 (1994); N. Gotoh *et al.*, *Oncogene* **11**, 2525 (1995); R. Nishikawa *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 7727 (1994)].
16. Others have observed a lack of correlation between MAP kinase activation and enhanced proliferation [M. Holgado-Madruga, D. R. Emlet, D. K. Moscatello, A. K. Godwin, A. J. Wong, *Nature* **379**, 560 (1996); C. J. Marshall, *Cell* **80**, 179 (1995)].
17. S. P. Soltoff and L. C. Cantley, *J. Biol. Chem.* **271**, 563 (1996).
18. P. C. Baas, G. M. DiGuglielmo, F. Authier, B. I. Posner, J. J. M. Bergeron, *Trends Cell Biol.* **5**, 465 (1995).
19. P. De Camilli, S. D. Emr, P. S. McPherson, P. Novick, *Science* **271**, 1533 (1996); C. Lamaze, T.-H. Chuang, L. J. Terlecky, G. M. Bokoch, S. L. Schmid,

Nature **382**, 177 (1996).
20. Surface biotinylation was performed according to the manufacturer's instructions with the use of NHS-LC-biotin (Pierce, Rockford, IL) at 40 nmol per 10^6 cells.
21. Cell extracts (1% Triton X-100) were immunoprecipitated [4 μg of immunoglobulin G (IgG) per 400 to 600 μg of lysate per 80 μl of immobilized Protein A/G or goat antibody to mouse IgG] and subjected to protein immunoblotting (1:2000 dilution of IgG) and ECL detection. Signals were quantitated with a Molecular Dynamics system.
22. Induced K44A and WT cells were serum-starved [0.4% serum in Dulbecco's modified Eagle's medium (DMEM)] for 20 hours and then cultured for 2 days in the presence or absence of 4 nM EGF. For the last 4 hours, MTT (Sigma) was added at 1 mg/ml and its reductive conversion was measured [F. Denizot and R. Lang, *J. Immunol. Methods* **89**, 271 (1986)]. For thymidine incorporation measurements, cells (~25% confluency) in DMEM + 10% fetal bovine serum (FBS) were incubated in serum-free DMEM, with or without tetracycline (1 μg/ml), for 40 hours. EGF (3.5 nM) was then added for 18 to 20 hours. Methyl-[^{3}H]thymidine (1 μCi/ml) (Amersham) was added for the last 4 hours. Cells were then processed as described [A. Obermeier, I. Tinhofer, H. H. Grunicke, A. Ullrich, *EMBO J.* **15**, 73 (1996)], and incorporated radioactivity was quantitated in the presence of ProteinPlus scintillant (Beckman).
23. Cells were seeded at ~20% confluency in DMEM + 10% FBS without tetracycline. After 36 hours, they were incubated in DMEM without serum for 16 to 20 hours. EGF (3.5 nM) was then added for 2 to 20 min, and the cells were rinsed three times with phosphate-buffered saline and lysed with MKAL buffer [0.5% Triton X-100, 20 mM Hepes, 100 mM NaCl, 200 μM sodium orthovanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, aprotinin (1 μg/ml), leupeptin (2 μg/ml), and 1 mM dithiothreitol (pH 7.4)]. Lysate (400 μg) was immunoprecipitated with 4 μg of affinity-purified anti-ERK IgG (UBI, catalog number 06-182). To the washed immunoprecipitates was added 1/10 volume of 10× assay buffer stock [200 mM Hepes, 500 mM NaCl, 1 mM sodium orthovanadate, 200 mM $MgCl_2$, 200 μM adenosine triphosphate (ATP), and 200 mM β-glycero-phosphate (pH 7.4)]. To each tube was added 2 μCi of γ-[^{32}P]ATP and myelin basic protein (MBP; 0.25 mg/ml), and the reactants were incubated at 32°C for 15 min. [^{32}P]MBP was then quantitated as described in Fig. 3.
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The Actin Cytoskeleton Is Required for Receptor-mediated Endocytosis in Mammalian Cells*

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Actin filament organization is essential for endocytosis in yeast. In contrast, the actin-depolymerizing agent cytochalasin D has yielded ambiguous results as to a role for actin in receptor-mediated endocytosis in mammalian cells. We have therefore re-examined this issue using highly specific reagents known to sequester actin monomers. Two of these reagents, thymosin β4 and DNase I, potently inhibited the sequestration of transferrin receptors into coated pits as measured in a cell-free system using perforated A431 cells. At low concentrations, thymosin β4 but not DNase I was stimulatory. Importantly, the effects of both reagents were specifically neutralized by the addition of actin monomers. A role for the actin cytoskeleton was also detected in intact cells where latrunculin A, a drug that sequesters actin monomers, inhibited receptor-mediated endocytosis. Biochemical and morphological analyses suggest that these reagents inhibit later events in coated vesicle budding. These results provide new evidence that the actin cytoskeleton is required for receptor-mediated endocytosis in mammalian cells.

The plasma membrane is directly linked to and functionally integrated with the underlying actin-based cytoskeleton which forms the cell "cortex." Thus, it might be anticipated that vesicular trafficking at the plasma membrane would require the active rearrangement of cortical actin filaments to remove a barrier to vesicular fusion or budding events. Alternatively, actin and actin-based motor proteins might be required to direct vesicle budding and fusion events through the cell cortex at the plasma membrane. Actin filaments can play both inhibitory and facilatory roles in exocytosis. For example, agonist-stimulated secretion appears to require localized disassembly of F-actin at the cell periphery (1, 2). Furthermore, low concentrations of proteins which sequester actin monomers can trigger regulated secretion in permeabilized pancreatic acinar cells suggesting that actin filaments might act as a clamp preventing fusion of docked vesicles (3). In contrast, higher concentrations of these actin-sequestering proteins inhibit regulated secretion, suggesting that actin filament integrity might also play an as yet undefined, facilatory role in regulated exocytosis (3).

Receptor-mediated endocytosis of the mating pheromone α-factor is potently inhibited in the yeast, *S. cerevisiae*, expressing mutations in either actin or the actin-binding protein, fimbrin (4). Yeast carrying mutations in three other genes, *END3*, *END5/VRP1*, and *END7/RVS167*, which disrupt actin organization, were also shown to be defective in endocytosis (5, 6). More recently a role for the type I myosin, myo5, in receptor-mediated endocytosis in yeast was revealed (7). Together these studies establish that actin filaments and actin-based motor proteins play an essential role in endocytosis in yeast.

In contrast, the role of actin in endocytosis in mammalian cells remains poorly understood. Cytochalasin D, a drug that destabilizes actin filaments, inhibits receptor-mediated and fluid-phase endocytosis at the apical surface of polarized Madin-Darby canine kidney cells (8) and Caco 2 cells (9), but has no effect on endocytosis at the basolateral surface. There are conflicting results on the effects of cytochalasin D on receptor-mediated endocytosis of transferrin in nonpolarized cells (10–13). Cytochalasin D caps the growing ends of actin filaments and thus causes the depolymerization of actin filaments that are actively turning over, *i.e.* predominantly the stress fibers. In contrast, cortical actin filaments are more resistant to disruption by cytochalasin D (15), providing a possible explanation for negative results using this reagent. Other lines of evidence have recently implicated actin filament organization in endocytosis. For example, activation of the Rho family GTPases, which trigger actin filament assembly at the cortex, has been shown to stimulate fluid phase pinocytosis (16, 17) but inhibit clathrin-mediated endocytosis (12). These findings prompted us to re-examine the role of actin microfilaments in receptor-mediated endocytosis using reagents which selectively sequester actin monomers, thereby disrupting actin filaments by shifting the equilibrium to the depolymerized state. Here we report that these actin-binding proteins or drugs inhibit the formation of clathrin-coated vesicles at the plasma membrane. These results provide new evidence that the actin cytoskeleton plays an essential role in receptor-mediated endocytosis in mammalian cells.

MATERIALS AND METHODS

Cells and Reagents—A431 cells were cultured in Dulbecco's modified Eagle's medium containing 10% defined fetal calf serum (Hyclone) as described previously (28, 29). Following trypsinization, 4×10^6 cells were seeded onto 15-cm culture dishes 20–24 h prior to preparing perforated cells, also as described previously (29). Biotinylated transferrin (B-Tfn)[1] was prepared (18, 29) using either sulfo-NHS-XX-biotin (6-((6-(biotinoyl)amino)hexanoyl)amino)hexanoic acid, sulfosuccinimidyl ester, sodium salt) obtained from Molecular Probes (Eugene, OR) or sulfo-NHS-SS-biotin (sulfosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate) from Pierce. The former biotinylating reagent was used, in general, for avidin sequestration assays as the longer spacer arm gave lower background signals. The latter cleavable biotinylating reagent was required for the MesNa (β-mercaptoethanesulfonic acid) assay.

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[1] The abbreviations used are: B-Tfn, biotinylated human diferric transferrin; TfnR, transferrin receptors; Tβ4, thymosin β4; MesNa, β-mercaptoethanesulfonic acid.

Thymosin β4 was expressed in *Escherichia coli* and purified to >99% homogeneity (based on Coomassie Blue-stained gels) as described (19). Monomeric actin was prepared from rabbit skeletal muscle (3). Latrunculin B was obtained from Alexis Corp. (San Diego, CA), latrunculin A was from Molecular Probes, DNase I was from Boehringer Mannheim, and cytochalasin D was from Sigma. All other materials were reagent grade.

Cell-free Assays for Receptor-mediated Endocytosis—Perforated A431 cells were prepared and assays were performed exactly as described previously (18, 29). For assays containing Tβ4 or DNase I, perforated cells were incubated with the drugs at 4 °C for 10 min before the addition of the cytosol. This treatment appeared to enhance the effects of DNase I and Tβ4. To block the effects of DNase I, actin monomers were added to the perforated cells at 4 °C for 10 min before the addition of DNase I. Tβ4 was inactivated *in vitro* by formation of Tβ4-actin complexes as described (3). Briefly, 7 μl of 160 μM Tβ4 was incubated with 42 μl of 180 μM G-actin (Tβ4:actin molar ratio of 1:5) for >1 h on ice and added to perforated cells.

Receptor-mediated Endocytosis in Intact Cells—Adherent A431 cells were dissociated for 5 min at 37 °C in phosphate-buffered saline containing 5 mM EDTA, washed in serum-free culture medium containing 0.2% bovine serum albumin and 20 mM Hepes, pH 7.2 (SFM), and resuspended at 2×10^7 cells/ml in SFM at 4 °C. Cells were then diluted 10-fold into SFM containing the indicated concentrations of latrunculin A or B (prepared as a 2 mg/ml stock solution and stored at 4 °C) and incubated at 37 °C for 1 h. Upon return to ice, B-Tfn was added (to 2 μg/ml) from a 20× stock. Aliquots (50 μl) were removed and kept on ice to determine total surface bound ligand and 4 °C controls, and the remaining suspension was returned to 37 °C. Aliquots were removed after increasing times to determine the kinetics of endocytosis. B-Tfn internalization was measured using the avidin protocol as described (20). Similar results were obtained if adherent cells were pretreated with latrunculin B before their release from the plate by phosphate-buffered saline/EDTA for endocytosis assays in suspension. Control experiments established that at the concentrations used, the solvent alone had no effect on endocytosis.

Electron Microscopy—Anti-human Tfn-R antibody HTR-D65 (obtained from I. Trowbridge, Salk Institute, La Jolla, CA) was conjugated with 10-nm gold particles (BBI International) as described previously (28). Incubations for morphological studies with intact cells were performed exactly as described for biochemical analysis except that they were scaled up 6-fold. After pelleting, cells were resuspended in 0.2 M cacodylate buffer (pH 7.2) containing 2% glutaraldehyde and processed for conventional epon sectioning as described (28). Samples were viewed on a Jeol 1200 at 60 kV. Quantitation of gold particles was performed at the microscope by random examination of cell profiles at a magnification of 20,000.

RESULTS AND DISCUSSION

Biphasic Effects of Thymosin β4 on Receptor-mediated Endocytosis—Thymosins are abundant and highly specific actin monomer-binding proteins ubiquitously expressed in vertebrate cells (21). Tβ4 forms a 1:1 complex with α-actin and sequesters actin monomers (22). As a result, actin monomers are not available for polymerization, and actin filaments are depolymerized. Although Tβ4 can directly interact with and depolymerize F-actin at high concentrations, at lower concentrations (<20 μM) it can neither sever nor cap actin filaments (23). Recent studies on the effects of Tβ4 on secretion in permeabilized acinar cells established the specificity of this reagent for disruption of actin filaments (3). Since Tβ4 disrupts actin filaments by a mechanism completely distinct from that of cytochalasin D, we tested its effects on receptor-mediated endocytosis in perforated A431 cells. Receptor-mediated endocytosis in this cell-free assay is dependent on cytosol and an ATP-regenerating system and is detected by the sequestration (either in constricted coated pits or sealed coated vesicles) of receptor-bound biotinylated ligands from exogenously added avidin (24, 29).

Titration of Tβ4 into perforated A431 cells showed that it had a biphasic effect on receptor-mediated endocytosis. Tβ4 concentrations up to 0.5 μM stimulated ligand sequestration by ~50% (Fig. 1, *A* and *inset*), whereas higher concentrations led to a complete inhibition of endocytosis (half-maximum inhibition at ~10 μM). Similar biphasic effects of Tβ4 were observed on secretion in permeabilized acinar cells (3). Importantly, the specificity of these effects could be verified by first inactivating Tβ4 *in vitro* with excess actin monomers before its addition to perforated cells. Under these conditions, both the activation of sequestration seen at low concentrations of Tβ4 and the inhi-

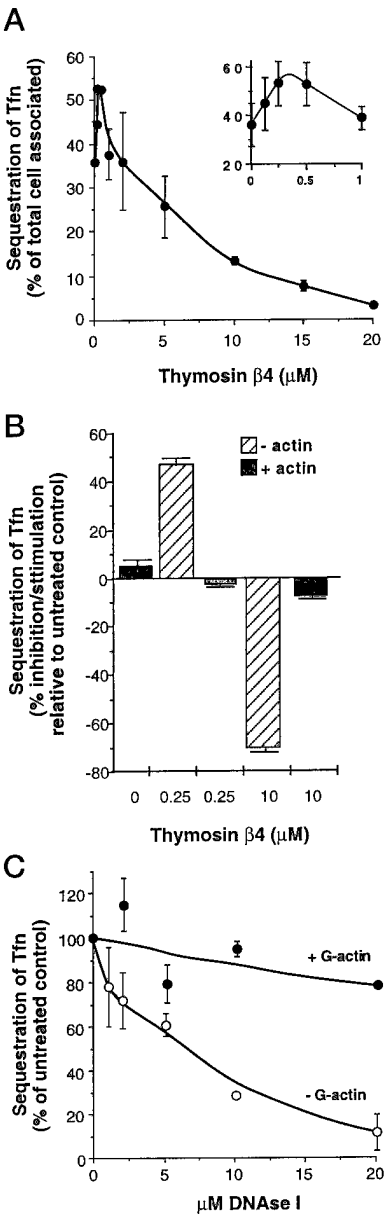

FIG. 1. **The actin-monomer-binding proteins thymosin β4 and DNase I inhibit receptor-mediated endocytosis in perforated cells.** The sequestration of B-Tfn into constricted coated pits or sealed coated vesicles was measured in perforated A431 cells by its inaccessibility to exogenously added avidin as described under "Materials and Methods." *Panel A*, assays were performed in the presence of the indicated concentrations of thymosin β4. The *inset* shows an expanded view of the stimulatory effects seen at low concentrations of Tβ4. ATP- and cytosol-dependent sequestration is expressed as a percentage of total cell-associated ligand. The data represent averages (± S.D.) of 9 experiments. *Panel B*, Tβ4, at the indicated concentrations, was preincubated with or without 5-fold molar excess of monomeric actin for 1 h at 4 °C prior to addition to perforated cells. Preincubation with actin abrograted both the stimulatory and inhibitory effects of Tβ4; actin on its own had no effect. The data represent averages (± S.D.) of five experiments. *Panel C*, sequestration assays were performed in the presence of the indicated concentrations of DNase I in the absence (○) or presence (●) 10 μM monomeric actin. The data represent averages (± S.D.) of three experiments.

bition seen at higher concentrations were abrograted (Fig. 1*B*). Exogenous actin on its own had no effect on receptor-mediated endocytosis. Together these results suggested involvement of the actin cytoskeleton in receptor-mediated endocytosis.

Effect of DNase I on Receptor-mediated Endocytosis—DNase I is a structurally distinct actin-sequestering protein with an exceptionally high affinity (K_d ~ 1 nM) for monomeric actin. DNase I is characterized by its unique ability to increase the depolymerization rate constant of actin at the pointed filament end without severing the actin filaments (25). As a result, the depolymerization of filaments capped at their barbed ends is accelerated, and actin monomers are sequestered. We therefore examined the effects of DNase I on receptor-mediated endocytosis in perforated cells and found that it also potently inhibited the sequestration of transferrin into coated pits (Fig. 1*C*, *open circles*). Inhibition by DNase I was concentration-dependent with half-maximal inhibition occurring at <5 μM. In contrast to Tβ4, no stimulation of endocytosis could be observed at low concentrations of DNase I. However, this could be explained by the difference in affinity for actin seen between DNase I and Tβ4 (~1 nM *versus* ~1 μM, respectively) and the differences in their effects on actin filament depolymerization. Again, the specificity of DNase I action on the actin network was confirmed since its inhibitory effects on endocytosis were neutralized in the presence of actin monomers (Fig. 1*C*, *closed circles*). Together with the effects seen with Tβ4, these results suggest that actin filaments are required for receptor-mediated endocytosis in perforated mammalian cells.

Effect of Latrunculins on Intact A431 Cells—The perforated A431 cell system has been extensively characterized both biochemically and morphologically and appears to faithfully reconstitute many of the biochemically distinct events required for the formation of endocytic clathrin-coated vesicles (12, 18, 20, 24, 28). Nonetheless, it remained possible that the requirement for actin assembly seen in perforated cells might reflect an artificial effect due to disorganization of the actin cytoskeleton as a result of the mechanical disruption of the plasma membrane. Therefore, we examined the effect of latrunculin A on receptor-mediated endocytosis in intact A431 cells. Latrunculins are a new class of membrane-permeable, actin-disrupting agents which show powerful and specific effects on the actin-based cytoskeleton of nonmuscle cells (26). *In vitro*, latrunculin affects the kinetics of polymerization of actin by forming a nonpolymerizable 1:1 molar complex with G-actin (27). Thus, like Tβ4 and DNase I but unlike the cytochalasins, latrunculins destabilize actin filaments by sequestering actin monomers and shifting the equilibrium to the disassembled state.

To determine whether actin filaments are required for receptor-mediated endocytosis in intact cells, A431 cells were exposed to increasing concentrations of latrunculin A for 1 h. This treatment induced dramatic changes in the morphology of adherent A431 cells which became round and contracted. As described previously (28) these morphological effects were similar to those seen when cells were incubated with 10 μg/ml cytochalasin D (data not shown). The morphology of A431 cells in suspension was not dramatically altered by latrunculin A treatment. The data in Fig. 2*A* show that latrunculin A significantly inhibited the rate of receptor-mediated endocytosis in intact cells in a concentration-dependent manner. The extent of inhibition (~50%) was consistent with those reported for the effects of cytochalasin D treatment in Hep2 cells (14). Half-maximal inhibition was obtained at ~4 μg/ml latrunculin A. While these concentrations are somewhat higher than those needed to destabilize stress fibers, they are not inconsistent with destabilization of more resistant elements of the cortical

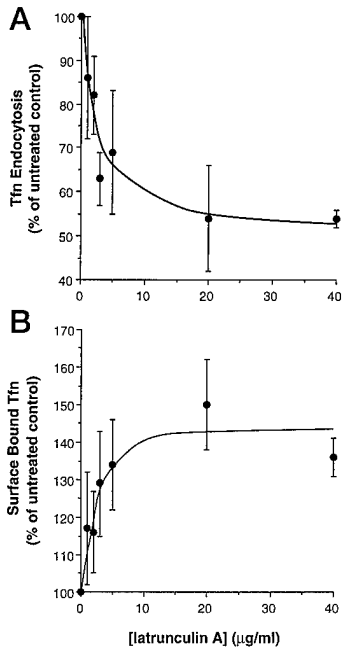

FIG. 2. **Latrunculin A inhibits receptor-mediated endocytosis and increases surface TfnR in intact cells.** Intact A431 cells in suspension were incubated with the indicated concentrations of latrunculin A for 60 min at 37 °C. B-Tfn (2 μg/ml) was then added on ice, and the kinetics of its internalization at 37 °C were determined as described under "Materials and Methods." *Panel A*, the results show the rates of endocytosis in latrunculin A-treated cells relative to untreated control cells. *Panel B*, the results show the total B-Tfn bound to the cell surface after 1 h at 4 °C. Averages (± S.D.) of three experiments.

actin network. Latrunculin B had similar effects although at slightly higher concentrations (not shown; but see below), consistent with this analogue being less potent. As previously shown (11, 12), cytochalasin D (10–50 μg/ml) had no effect on endocytosis in A431 cells.

TfnR undergo constitutive endocytosis and recycling. As a result, the number of surface TfnR reflects the relative rates of internalization and recycling. As can be seen in Fig. 2*B*, cells incubated with latrunculin A show a concentration-dependent increase in surface TfnR. Thus, the effects of latrunculin A on intact cells are consistent with an inhibition in endocytosis without a concomitant effect on TfnR recycling. A similar finding was reported following cytochalasin D treatment in Hep2 cells (14). Together, these results confirm a requirement for actin in receptor-mediated endocytosis both in intact and perforated mammalian cells.

Actin Filaments Are Not Required for Clustering of TfnR into Coated Pits—Efficient receptor-mediated endocytosis requires both the concentration of receptor-bound ligands into coated pits and the subsequent budding of coated vesicles. Thus, it remained possible that coated vesicle formation continued in the presence of these actin-monomer sequestering agents, but TfnR clustering in coated pits was impaired. To test this we used the gold-conjugated anti-TfnR monoclonal antibody D65 and conventional thin section electron microscopy to examine the distribution of TfnR relative to coated pits in A431 cells treated with or without 25 μM latrunculin B. This treatment resulted in a 40% inhibition of TfnR endocytosis measured in parallel using biochemical assays. The micrographs in Fig. 3 show that neither the morphology of coated pits nor their ability to cluster TfnR was affected. Quantitation of these results showed that 37% of surface D65-gold was found associated with coated pits in both control (100 of 270 gold particles counted) and latrunculin B-treated (114 of 308 gold particles counted) samples.

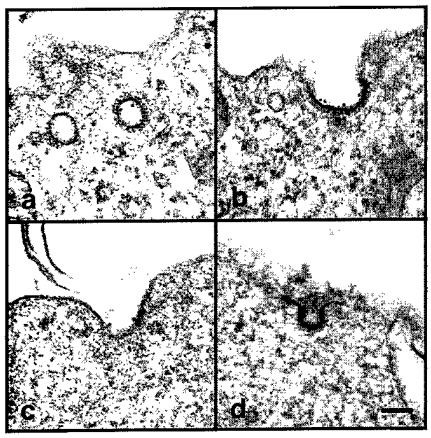

FIG. 3. **TfnR clustering into coated pits is unaffected by latrunculin B.** Electron micrographs of coated pits from A431 cells incubated without (*a* and *b*) or with (*c* and *d*) 25 μg/ml latrunculin B at 37 °C. Cells were then incubated with gold-conjugated anti-TfnR monoclonal antibody D65 for 60 min on ice before washing, fixation, and processing for conventional epon sections. The *bar* represents 100 nm.

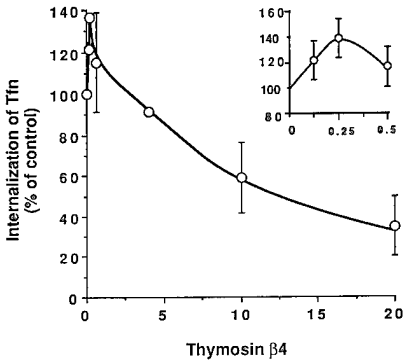

FIG. 4. **Thymosin β4 affects late events in coated vesicle formation.** The internalization of BSS-Tfn into sealed coated vesicles was measured in perforated A431 cells using the MesNa assay as described under "Materials and Methods." Assays were performed in the presence of increasing concentrations of Tβ4. The *inset* shows an expanded view of the stimulatory effects seen at low concentrations of Tβ4. The data represent averages (± S.D.) of four experiments.

Actin Filaments Are Required for Clathrin-coated Vesicle Budding—Confirmation that actin filaments were required for coated vesicle formation was obtained using the perforated cell assay system to selectively measure the budding of preformed coated pits. Previous characterization of this system has established that detachment of preformed coated pits can be measured selectively using the small membrane-impermeant reducing agent MesNa as a probe for the internalization of biotinylated ligands into sealed coated vesicles (24, 28). Using this assay the formation of constricted coated pits and coated vesicle budding are detected when receptor-bound biotinylated ligands are internalized into sealed vesicles that are inaccessible to MesNa. Using the MesNa assay, we again found a biphasic response to thymosin β4. The data in Fig. 4 show that ligand internalization, like sequestration, was stimulated at low concentrations and inhibited at higher concentrations of Tβ4. The inhibition seen at high concentrations of Tβ4 is consistent with the results of ultrastructural analysis and suggest that actin filaments are required for late events (either for coated pit constriction, coated vesicle detachment or both) in endocytic coated vesicle formation. The stimulation of coated vesicle budding seen at low concentrations of Tβ4 could reflect destabilization of actin filaments that otherwise act as a barrier to vesicle budding and detachment as observed for exocytosis (3).

In summary, the use of highly specific actin modulatory proteins that sequester actin monomers has revealed a requirement for actin filaments in receptor-mediated endocytosis in mammalian cells. These results resolve apparent discrepancies and suggest a similarity in the mechanisms of receptor-mediated endocytosis in yeast and mammalian cells. It will be important to determine whether the actin requirement in mammalian cells reflects a direct involvement of actin filaments in coated vesicle budding or instead reflects a more general requirement for the structural integrity of the cell cortex in plasma membrane function. Additional evidence for the involvement of other actin binding proteins or actin-based type I myosins, as appears to be the case for endocytosis in yeast (4, 7), may help to distinguish these two possibilities.

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REFERENCES

1. Vitale, M. L., Rodrigues del Castillo, A., Tchakarov, L., and Trifaró, J.-M. (1991) *J. Cell Biol.* **113,** 1057–1067
2. Trifaró, J.-M., Rodrigues del Castillo, A., and Vitale, M. L. (1992) *Mol. Neurobiol.* **6,** 339–358
3. Muallem, S., Swiatkowska, K., Xu, X., and Yin, H. L. (1995) *J. Cell Biol.* **128,** 589–598
4. Kübler, E., and Riezman, H. (1993) *EMBO J.* **12,** 2855–2862
5. Bénédetti, H., Raths, S., Crausaz, F., and Riezman, H. (1994) *Mol. Biol. Cell* **5,** 1023–1037
6. Munn, A. L., Stevenson, B. J., Gelei, M. I., and Riezman, H. (1995) *Mol. Biol. Cell* **6,** 1721–1742
7. Geli, M. I., and Riezman, H. (1996) *Science* **272,** 533–535
8. Gottlieb, T. A., Ivanov, I. E., Adesnic, M., and Sabatini, D. D. (1993) *J. Cell Biol.* **120,** 695–710
9. Jackman, M. R., Shurety, W., Ellis, J. A., and Luzio, J. P. (1994) *J. Cell Sci.* **107,** 2547–2556
10. Wolkoff, A. W., Klausner, R. D., Ashwell, G., and Harford, J. (1984) *J. Cell Biol.* **98,** 375–381
11. Sandvig, K., and van Deurs, B. (1990) *J. Biol. Chem.* **265,** 6382–6388
12. Lamaze, C., Chuang, T.-H, Terlecky, L. J., Bokoch, G. M., and Schmid, S. L. (1996) *Nature* **382,** 177–179
13. Salisbury, J. L., Condeelis, J. S., and Satir, P. (1980) *J. Cell Biol.* **87,** 132–141
14. Durrbach, A., Louvard, D., and Coudrier, E. (1996) *J. Cell Sci.* **109,** 457–465
15. Cassimeris, L., Shafer, D., Nachmias, V. T., and Zigmond, S. H. (1990) *J. Cell Biol.* **110,** 1067–1075
16. Schmalzing, G., Richter, H.-P., Hanse, A., Schwartz, W., Just, I., and Aktories, K. (1995) *J. Cell Biol.* **130,** 1319–1332
17. Ridley, A. J. (1996) *Curr. Biol.* **6,** 1256–1264
18. Carter, L. L., Redelmeier, T. E., Woollenweber, L. A., and Schmid, S. L. (1993) *J. Cell Biol.* **120,** 37–45
19. Yu, F.-X., Lin, S.-C., Morrison-Bogorad, M., Atkinson, M. A. L., and Yin, H. L. (1993) *J. Biol. Chem.* **268,** 502–509
20. Lamaze, C., and Schmid, S. L. (1995) *J. Cell Biol.* **129,** 47–54
21. Sun, H.-Q., Kwiatkowska, K., and Yin, H. L. (1995) *Curr. Opin. Cell Biol.* **7,** 102–110
22. Weber, A., Nachmias, V. T., Pennise, C. R., Pring, M., and Safer, D. (1992) *Biochemistry* **31,** 6179–6185
23. Carlier, M.-F., Didry, D., Erk, I., Lepault, J., Van Troys, M. L., Vandekerckhove, J., Perelroizen, I., Yin, H., Doi, Y., and Pantaloni, D. (1996) *J. Biol. Chem.* **271,** 9231–9239
24. Schmid, S. L. (1993) *Trends Cell Biol.* **3,** 145–148
25. Weber, A., Pennise, C. R., and Pring, M. (1994) *Biochemistry* **33,** 4780–4786
26. Spector, I., Shochet, N. R., Blasberger, D., and Kashman, Y. (1989) *Cell Motil. Cytoskeleton* **13,** 127–144
27. Coué, M., Brenner, S. L., Spector, I., and Korn, E. D. (1987) *FEBS Lett.* **213,** 316–318
28. Schmid, S. L., and Smythe, E. (1991) *J. Cell Biol.* **114,** 869–880
29. Smythe, E., Redelmeier, T. E., and Schmid, S. L. (1992) *Methods Enzymol.* **219,** 223–234